RESEARCH ARTICLE

The Clinical Course of Experimental Autoimmune Encephalomyelitis is Associated with a Profound and Sustained Transcriptional Activation of the Genes Encoding Toll-like Receptor 2 and CD14 in the Mouse CNS

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Experimental autoimmune encephalomyelitis (EAE) is an autoimmune demyelinating disease commonly used to model the pathogenetic mechanisms involved in multiple sclerosis (MS). In this study, we examined the effects of immunization with the myelin oligodendrocyte glycoprotein MOG_{35-55} on the expression of molecules of the innate immune system, namely toll-like receptor 2 (TLR2) and CD14. Expression of the mRNA encoding TLR2 increased in the choroid plexus, the leptomeninges and within few isolated cells in the CNS parenchyma 4 to 8 days after immunization with MOG. At day 10, the signal spread across the meninges, few perivascular regions and over isolated groups of parenchymal cells. Three weeks after the MOG treatment, at which time animals showed severe clinical symptoms, a robust expression of both TLR2 and CD14 transcripts occurred in barrier-associated structures, as well as parenchymal elements of the spinal cord, and within numerous regions of the brain including, the medulla, cerebellum and the cortex. Dual labeling provided the anatomical evidence that microglia/macrophages were positive for TLR2 in the brain of EAE mice. The regions that exhibited chronic expression of TLR2 and CD14 were also associated with an increase in NF-κB activity and transcriptional activation of genes encoding numerous proinflammatory molecules. The present data provide evidence that receptors of the pathogen-associated molecular patterns are strongly induced in the CNS of EAE mice, further reinforcing the concept that the innate immune system plays a determinant role in this autoimmune demyelinating disease.

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Introduction

The innate immune response is characterized by a robust and transient induction of proinflammatory molecules that are together involved in controlling and restraining the invading pathogens. Host organisms detect the presence of infection by recognizing specific elements produced by microorganisms, such as Gram-negative bacteria, Gram-positive bacteria, and mannans of fungi (1). These elements, referred to as pathogen-associated molecular patterns (PAMPs), are recognized by specific cells of the immune system to activate innate mechanisms which then mount a rapid response to bacterial infection, and in many cases require activation of the transcription factor NF-κB. The recent characterization of human homologues of Toll has provided the missing link for the transduction events leading to NF-κB activity and cytokine production in response to the bacterial cell wall components (4). A large family of Toll-like receptors (TLRs) has already been characterized, sharing similar extracellular and cytoplasmic domains (1).

Some of these events are also taking place in the brain; TLR4 mRNA is constitutively expressed in the circumventricular organs (CVOs)—brain regions that contain a rich vascular plexus with specialized arrangements of the blood vessels (10). Circulating LPS also causes a rapid increase in CD14 (8), TLR2 (11), cytokines (14), chemokines (17) and members of the complement system (13) in these leaky structures. A delayed response can be found in parenchymal cells located in the anatomical boundaries of the CVOs, and thereafter, in microglia across the brain parenchyma. The basal expression of CD14 and TLR4 in the CVOs is likely to be a determinant mechanism in the proinflammatory signal transduction events that originate from these structures during the innate immune response. Indeed, cell wall components of the Gram-negative bacteria may be selectively recognized by the TLR4/CD14-bearing cells of the CVOs, which allows the LPS signaling and the subsequent rapid transcription of

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proinflammatory cytokines first within these organs, and thereafter, across the brain parenchyma during severe endotoxemia.

The physiological relevance of the cerebral innate immune response remains unknown. It may be involved in orchestrating the inflammatory response that takes place within and across the parenchymal microglia, the resident macrophages of the brain. It is possible that these cells have the ability to express receptors of the innate immunity not only during bacterial infections, but also during neurological disorders that have an immune etiology. Here we determined the expression pattern of the genes encoding these innate molecules during the progression of the demyelinating disease experimental autoimmune encephalomyelitis (EAE), an animal model commonly used to examine the pathogenetic mechanisms of human multiple sclerosis (MS) (12). Immunization of C57BL/6 mice with an encephalitogenic peptide of the myelin oligodendrocyte glycoprotein (MOG_{35-55}) caused a long lasting disease characterized by leukocyte infiltration, inflammatory gene expression, and subsequent demyelination (6). We now report that the development of MOG-induced EAE in mice is associated with a profound transcriptional activation of CD14 and TLR2 within parenchymal microglial cells, suggesting that the innate immune response may play a role in the etiology of this disease.

Materials and Methods

Animals. Female C57BL/6 mice, aged 6 to 8 weeks, were purchased from Charles River Breeding (Cambridge, MA). Mice were kept under controlled 12:12 hour light/dark conditions and fed ad lib. All experiments were carried out with approval of the local animal welfare committee for animal experiments.

Induction of EAE. EAE was actively induced using synthetic myelin oligodendrocyte glycoprotein peptide 35-55 (MOG_{35-55}) as described (5). The MOG_{35-55} peptide (MEVGWYRSPFSRVVHLYRNGK) was synthesized by Dr Suzanna J. Horvath at the Biopolymer Synthesis Center (California Institute of Technology, Pasadena, Calif) and purified to >98% purity by reverse phase HPLC analysis. Mice were injected subcutaneously (two 100 μl injections into adjacent areas in one hind limb) with an emulsion of 300 μg MOG_{35-55} dissolved in 100 μl PBS, mixed with 100 μl CFA containing 500 μg of Mycobacterium tuberculosis (Difco, Detroit, Mich). Some animals were immunized as above but without MOG_{35-55} peptide. Immediately after MOG_{35-55} injection, the animals received an i.p. injection of pertussis toxin (PT, 200 ng in 200 μl PBS). Two days later the mice received a second PT injection and 1 week later they received a booster injection of MOG_{35-55} (referred to as day 0).

In situ hybridization histochemistry. During the progression of the disease, mice of each group were deeply anesthetized and then rapidly perfused transcardially with 0.9% cold saline, followed by 4% paraformaldehyde in 0.1 M borax buffer (pH 9.5 at 4°C). Brains and spinal cords were removed, postfixed for 2 to 8 days and then placed in a solution containing 10% sucrose diluted in 4% paraformaldehyde-borax buffer overnight at 4°C. The frozen brains and spinal cords were mounted on a microtome (Reichert-Jung, Cambridge Instruments Company, Deerfield, Ill) and cut into 20-μm coronal and longitudinal (spinal cord) sections. The slices were collected in a cold cryoprotectant solution (0.05 M sodium phosphate buffer, pH 7.3, 30% ethylene glycol, 20% glycerol) and stored at -20°C.

Hybridization histochemical localization of TLR2, CD14, TNF-α, IκBα and MCP-1 transcripts was carried out on every sixth section of the brains and spinal cords using ^{35}S-labeled cRNA probes. All solutions were treated with diethylpyrocarbonate (DEPC) and sterilized to prevent RNA degradation. Tissue sections mounted onto poly-L-lysine-coated slides were desiccated overnight under vacuum, fixed in 4% paraformaldehyde for 30 minutes, and digested with proteinase K (10 μg/ml in 0.1 M tris HCl, pH 8.0, and 50 mM EDTA, pH 8.0, at 37°C for 25 minutes). Thereafter, brain and spinal cord sections were rinsed in sterile Depc water followed by a solution of 0.1 M triethanolamine (TEA, pH 8.0), acetylated in 0.25% acetic anhydride in 0.1 M TEA, and dehydrated through graded concentrations of alcohol (50, 70, 95, and 100 %). After vacuum drying for a minimum of 30 minutes, 90 μl of hybridization mixture (10^7 cpm/ml) was spotted on each slide, sealed under a coverslip, and incubated at 60°C overnight (~15-20 hours) in a slide warmer. Coverslips were then removed and the slides were rinsed in 4× standard saline citrate (SSC) at room temperature. Sections were digested by RNAse A (20 μg/ml, 37°C, 30 minutes), rinsed in descending concentrations of SSC (2×, 1×, 0.5× SSC), washed in 0.1× SSC for 30 minutes at 60°C (1× SSC: 0.15 M NaCl, 15 mM trisodium citrate buffer, pH 7.0) and dehydrated through graded concentrations of alcohol. After being dried for 2 hours under vacuum, the sections were exposed at 4°C to X-ray films (Biomax, Kodak, Rochester, NY) for 1 to 3 days. The slides were

Plasmid	Vector	Length (bp)	Enzymes used for the sense probe	Enzymes used for the antisense probe	Source
mouse TLR2	PCR-blunt II-topo	2278 (almost full)	*Spe*/T7	*Eco*R V/Sp6	cloned by PCR
rat CD14	PBluescript SK	1528 (full)	*Kpn* I/T3	*Sac* I/T7	Dr. Doug Feinstein, University of Illinois, Chicago, United States
mouse IκBα	Bluescript SK II+	1114 (full)	*Hind* III/T3	*Bam*H I/T7	Dr. A. Israël, Institut Pasteur, Paris, France
mouse MCP-1	pGEMEX-1	598	*Sac* I/SP6	*Bam*H I/T7	Dr Simon C. Williams Texas Tech University, United States
mouse TNF-α	Bluescript K II+	1300 (full+a 593bp non-coding part)	*Bam*H I/T7	*Pst* I/T3	Subcloned from a PUc19 plasmid provided by Dr. D. Radzioch McGill Univ., Montréal, Canada

Table 1. Plasmids and enzymes used for the synthesis of the cRNA probes.

then defatted in xylene, dipped in NTB-2 nuclear emulsion (Kodak; diluted 1:1 with distilled water), exposed for 17 days (TNF-α transcript), 16 days (CD14 transcript), 12 days (TLR2 and MCP-1 transcripts), or 8 days (IκBα transcript). The slides were then developed in D19 developer (Kodak) for 3.5 minutes at 14 to 15°C, washed 15 seconds in water, and fixed in rapid fixer (Kodak) for 5 minutes. Tissues were then rinsed in running distilled water for one hour, counterstained with thionin (0.25%), dehydrated through graded concentrations of alcohol, cleared in xylene, and coverslipped with distrene plasticizer xylene (DPX) mounting medium (Electron Microscopy Science, Washington, Pa).

cRNA probe synthesis and preparation. Plasmids were linearized and the sense and antisense riboprobes synthesized as described in Table 1. Radioactive cRNA copies were synthesized by incubation of 250 ng linearized plasmid in 6 mM MgCl2, 40 mM Tris (pH 7.9), 2 mM spermidine, 10 mM NaCl, 10 mM dithiothreitol, 0.2 mM ATP/GTP/CTP, 100 μCi of α-^{35}S-UTP (Dupont NEN, #NEG 039H), 20 U RNAsin (Promega, Madison, WI) and 10U of either T7, SP6 or T3 RNA polymerase for 60 minutes at 37°C (see Table 1). Unincorporated nucleotides were removed using ammonium-acetate precipitation method; 100 μl of DNAse solution (1 μl DNAse, 5 μl of 5 mg/ml tRNA, 94 μl of 10 mM tris/10 mM MgCl2) was added, and 10 minutes later, a phenol-chloroform extraction was performed. The cRNA was precipitated with 80 μl of 5 M ammonium acetate and 500 μl of 100% ethanol for 20 minutes on dry ice. The pellet was dried and resuspended in 50 μl of 10 mM Tris/1 mM EDTA. A concentration of 10^7 cpm probe was mixed into 1 ml of hybridization solution (500 μl formamide, 60 μl 5 M NaCl, 10 μl 1 M Tris [pH 8.0], 2 μl 0.5 M EDTA [pH 8.0], 50 μl 20× Denhart's solution, 200 μl 50% dextran sulfate, 50 μl 10 mg/ml tRNA, 10 μl 1M DTT, [118 μl Depc water—volume of probe used]). This solution was mixed and heated for 10 minutes at 65°C before being spotted on slides.

Combination of immunocytochemistry with in situ hybridization. Immunocytochemistry was combined with the in situ hybridization protocol to determine the type(s) of cells that express TLR2 transcript in the mouse brain after immunization with MOG_{35-55}. Among the antibodies selected for this study, anti-Von Willebrand Factor (vWF) was used to stain the endothelial cells of the microvasculature, anti-glial fibrillary acidic protein (GFAP) stained the astrocytes, whereas anti-ionized calcium binding adapter molecule 1 (*iba1*) labeled cells of myeloid lineage (macrophages and microglia). Every fifth brain section was processed by using the avidin-biotin amplification bridge method with peroxidase as a substrate. Slices were briefly washed in sterile Depc-treated 50 mM potassium phosphate-buffered saline (KPBS) and incubated for 2 hours at room temperature with either vWF, *iba1*, or GFAP antibody diluted in

sterile KPBS *plus* 0.5% Triton X-100 *plus* 1% bovine serum albumin (BSA, fraction V, Sigma, St. Louis, Mo) *plus* 0.25% heparin sodium salt USP (ICN Biomedicals Inc., Aurora, Ohio). Sheep anti-vWF (Cederlane Laboratory limited, Canada, Catalog# CL20176A-R, Lot# AB22-74), rabbit anti-iba1 (generously provided by Dr Y. Imai, National Institute of Neuroscience, Kodaira, Tokyo, Japan; (9) and anti-GFAP monoclonal antibody raised in mouse (Chemicon, Temecula, Calif, Catalog# MAB360, Lot# 19020465) were diluted at 1:1000, 1:2000, and 1:3000, respectively. After incubation with the primary antibodies, brain slices were rinsed in sterile KPBS and incubated with a mixture of KPBS *plus* 0.4% triton-X *plus* 1% BSA *plus* 0.25% heparin *plus* biotinylated secondary antibodies (rabbit anti-sheep IgG for vWF, goat anti-rabbit for iba1 and horse anti-mouse IgG for GFAP, 1:1500 dilution; Vector Laboratories, Calif) for 60 minutes. Sections were then rinsed with KPBS and incubated at room temperature for 60 minutes with an avidin-biotin-peroxidase complex (Vectastain ABC elite kit, Vector Laboratories, Calif). After several rinses in sterile KPBS, the brain slices were reacted in a mixture containing sterile KPBS, the chromagen 3,3′-diaminobenzidine tetrahydrochloride (DAB, 0.05%) and 0.003% hydrogen peroxide (H_2O_2).

Thereafter, tissues were rinsed in sterile KPBS, immediately mounted onto gelatine and poly-L-lysine-coated slides, desiccated under vacuum for 30 minutes, fixed in 4% paraformaldehyde for 20 minutes and digested by proteinase K (10 μg/ml in 100 mM tris HCl [pH 8.0] and 50 mM EDTA [pH 8.0]) at 37°C for 25 minutes. Prehybridization, hybridization, and posthybridization steps were performed as describe above with the difference of shortened dehydration dip (alcohol 50, 70, 95, 100%) to avoid decoloration of the brown immunoreactive staining cells. The presence of the TLR2 transcripts was detected by the agglomeration of silver grains in perikarya, whereas vWF, *iba1*, and GFAP immunoreactivity within the cell cytoplasm and ramifications (microglia, astrocytes) was indicated by a brown homogeneous coloration. Determination of the double-labeled cells was performed visually for each cell exhibiting clear brown cytoplasm and a number of silver grains within the cell body delineating convincing hybridized message.

Results

MOG-induced receptors of the innate immune system in the CNS. Figure 1 shows the expression pattern of the gene encoding TLR2 in the brain of mice killed at different times after being immunized with MOG_{35-55}. As previously reported, the choroid plexus (chp) displayed a positive TLR2 hybridization signal under basal conditions (11). This constitutive expression was found in the chp lining the lateral ventricle, surrounding the subfornical organ (SFO), at the edge of the fimbria, stria terminalis and optic tract, as well as at the level of the inferior cerebellar peduncle. Such basal expression remained quite similar in mice that were killed 1 to 4 days after being immunized or not with the synthetic peptide (Figure 1, left column). In these animals, there was no positive signal in any structures of the brain parenchyma.

A significant increase of the TLR2 hybridization signal occurred in restricted regions of the CNS at day 8 post immunization. This was particularly convincing in the chp and leptomeninges underneath the optic tract and the periamygdaloid cortex as well as those lining the cochlear nucleus (Figure 1, second column from the left). TLR2-expressing cells were also found in isolated fissures of the cerebellum and the area postrema. At day 10 the signal increased in these barrier-associated regions, especially in the chp lining the stria terminalis, the optic tract, and the lateral nucleus of the amygdala. Of interest is the expression of the innate receptor in localized elements of the brain parenchyma at that time. These groups of cells were associated with the microvasculature that became positive 10 days following immunization with MOG_{35-55}, but this phenomenon was variable among animals. Some animals failed to exhibit TLR2-expressing cells along the endothelium of the brain capillaries and their surrounding parenchymal elements, whereas others had very few and localized inflammatory clusters.

This contrasts with the robust hybridization signal for the gene encoding the innate receptor across the CNS of mice that were killed at 3 weeks when the animals demonstrated severe signs of sickness. The message was very strong in most of the previously described regions as well as within parenchymal elements in periphery of the microvasculature (Figure 1, right column). However, numerous isolated cells not associated with the endothelium of the brain capillaries were also found across the CNS parenchyma (both in the brain and spinal cord) of mice killed 3 weeks after being immunized with the antigenic peptide.

Dual-labeling that combined immunocytochemistry with in situ hybridization on the same sections provided the anatomical evidence that the mRNA encoding TLR2 was essentially expressed within cells of myeloid origin. Indeed, macrophages and microglia that were

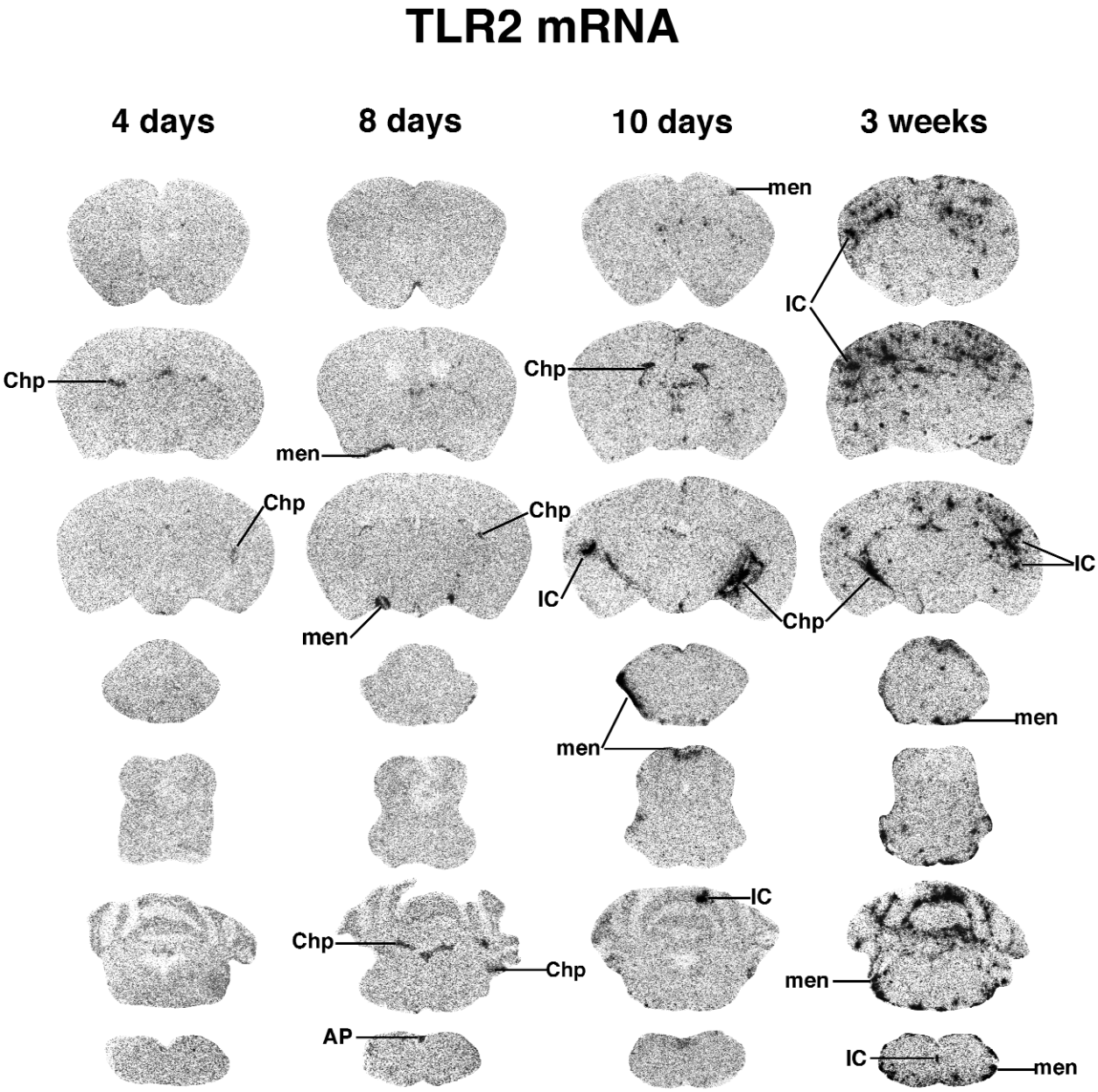

Figure 1. Time-related expression of the gene encoding TLR2 across the brain of mice immunized with the oligodendrocyte glycoprotein MOG_{35-55}. These rostrocaudal sections (20 μm) depict a positive signal on x-ray films (Biomax, Kodak) for the TLR2 transcript in various structures of the brain of animals killed 4, 8, 10, or 21 days after the booster injection of MOG. Note the strong increase of TLR2 mRNA at 10 days within the meninges and the choroid plexus. The signal was intense throughout the cerebral tissue of animals killed 3 weeks in the course of EAE. AP, area postrema; Chp, choroid plexus; IC, inflammatory cluster; men, meninges.

stained with an antisera directed against *iba1* exhibited agglomeration of silver grains delimiting positive TLR2 cells (Figure 2). In contrast, TLR2-expressing cells rarely overlapped with those immunoreactive for GFAP, despite the presence of astrogliosis in the CNS of EAE mice at 3 weeks post immunization (Figure 2, bottom right). A lack of positive signal for TLR2 mRNA was also found over cells that were immunoreactive to the vWF (data not shown).

The LPS receptor CD14 is up-regulated during the course of EAE. The expression pattern of CD14 was

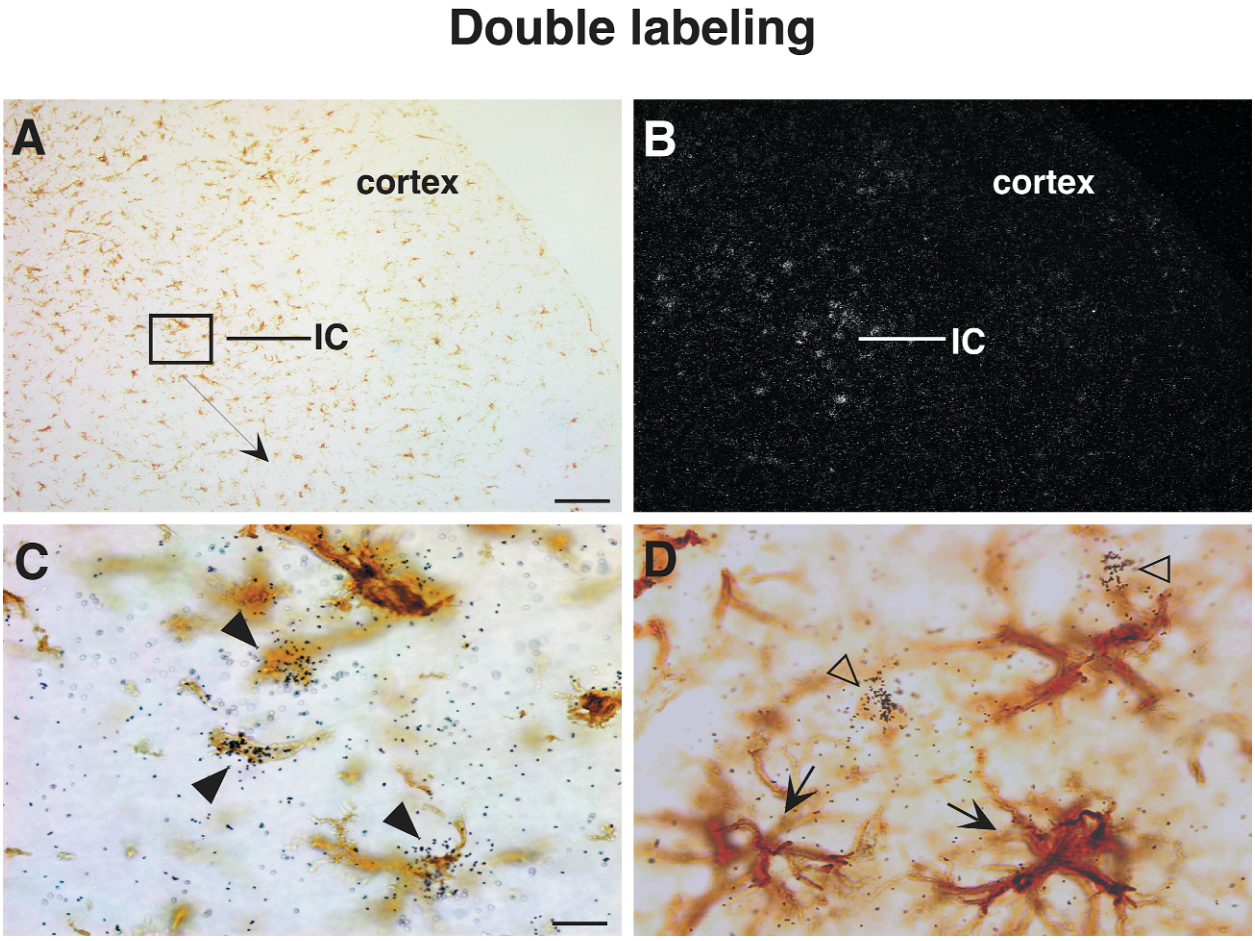

Figure 2. Colocalization of TLR2-expressing cells within the parenchymal microglia of EAE animals. Cells of myeloid origin were labeled by immunoperoxidase using an antisera directed against the ionized calcium binding adapter molecule 1 (*iba1*, **A-C**). The anti-glial GFAP monoclonal antibody was used to stain astrocytes (panel D). TLR2 mRNA was thereafter hybridized on the same sections by means of radioactive in situ hybridization (silver grains). **A.** A low magnification of iba1-immunoreactive cells in the cerebral cortex. **B.** Darkfield photomicrograph of the same section as panel A. **C.** Depicts high magnification of cells that are immunoreactive for *iba1* (brown immunoreactive cells) and positive for the mRNA encoding TLR2 (silver grains into the cytoplasm, filled arrowheads). **D.** Shows the lack of colocalization between GFAP-immunoreactive cells (arrows) and TLR2-expressing cells (open arrowheads). IC, inflammatory cluster. Magnification **A** and **B**, 10× (scale bar, 250 μm); C and D, 100× (scale bar, 10 μm).

quite similar except for the presence of positive cells lining few blood vessels of mice killed at day 4 post immunization. Four days later, a strong hybridization signal for the LPS receptor was found in the leptomeninges at the edge of the optic tract and within the chp (Figure 3). The signal increased in the meningeal formation and along the penetrating arterioles, capillaries, and small venules across the CNS of mice killed 10 days after the immunization with MOG_{35-55}. At that time, small-scattered CD14 cells were also detected in the CNS parenchyma. As for the TLR2 transcript, the mRNA encoding CD14 was widely expressed in the brain and spinal cord of EAE mice 3 weeks after the treatment, whereas animals that received CFA without the antigenic peptide had only a constitutive expression in few blood vessels, chp and the meninges.

Proinflammatory signaling and cytokine/chemokine induction. De novo expression of the inhibitory factor kappa B alpha (IκBα) mRNA reflects the activity of NF-κB (9, 10, 16), which is the critical transcription factor for most proinflammatory cytokines and chemokines involved in the innate immune response. As depicted in Figure 4, treatment with CFA alone had the ability to trigger IκBα expression along the endothelium of the large blood vessels and small capillaries.

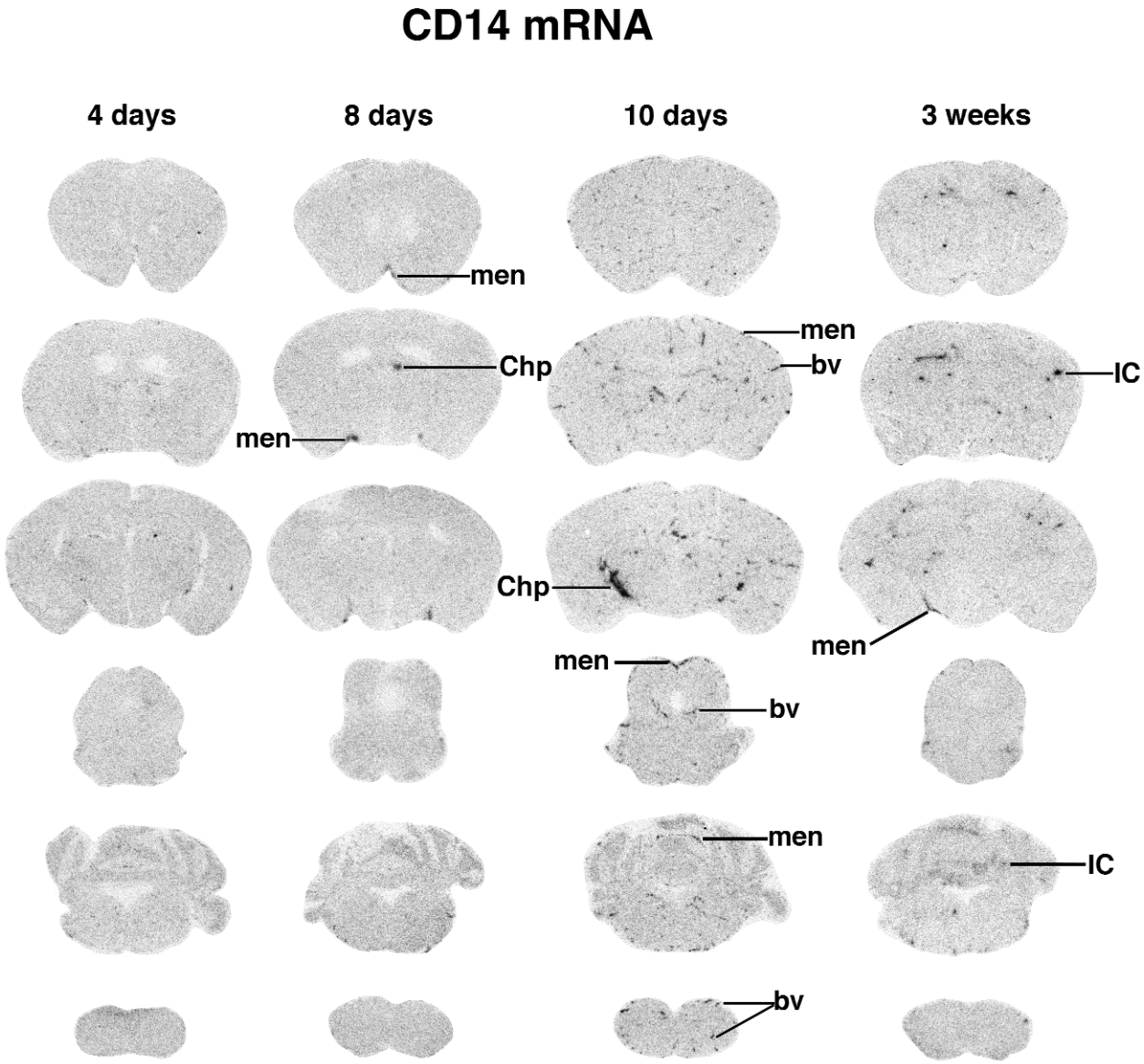

Figure 3. Cerebral expression of the LPS receptor CD14 mRNA during the course of EAE. These rostrocaudal coronal sections (20 μm) depict positive hybridization signal on x-ray films (Biomax, Kodak) in the brain at 4, 8, 10, or 21 days post MOG immunization. From day 10, a strong increase in CD14 mRNA levels occurred in cells lining the blood vessels and meninges. For abbreviations, see Figure 1.

However, the hybridization signal rarely extended to the parenchymal elements of the CNS, which differed from the cerebral tissues of mice that were immunized with MOG_{35-55}. Indeed, a robust signal spread across the brain parenchyma in the course of EAE indicating that activation of the NF-κB signaling occurred in these cells in response to the antigenic peptide.

Such activity of the proinflammatory signal transduction pathway is further reinforced by the parallel increases in both TNF and MCP-1 transcripts in the same groups of cells. These 2 molecules play crucial roles in the MS pathogenesis and are rapidly induced not only in structures that can be reached by the bloodstream, but also in the localized surfaces of the brain parenchyma. At day 10, isolated groups of cells expressing the gene encoding TNF were already found in the cortex and spinal cord of EAE mice (Figure 5). However, these clusters of TNF cells were mostly limited to the cerebral cortex and the spinal cord, although a few isolated cells were also present within the brain

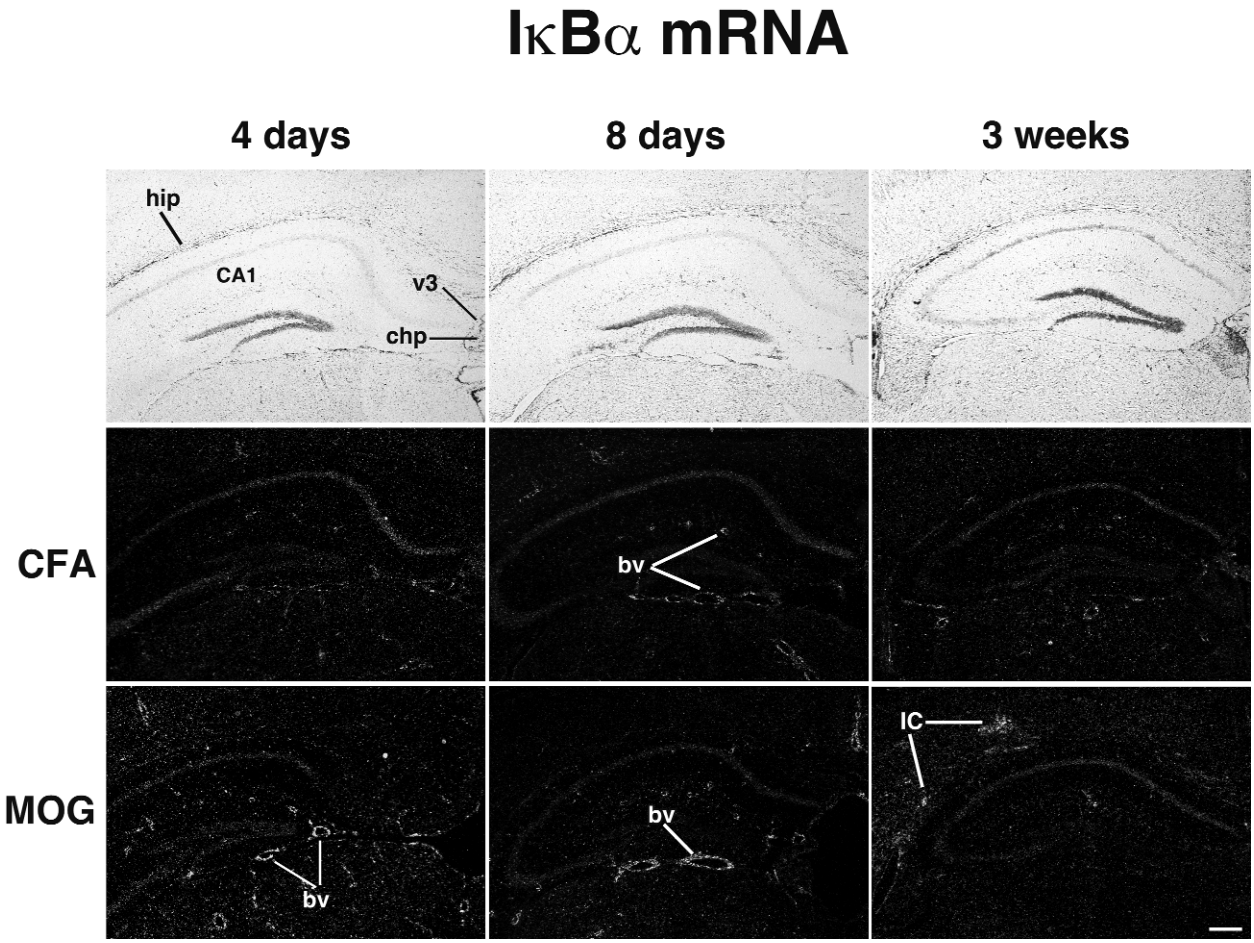

Figure 4. NF-κB activity (as revealed by IκBα transcriptional activation) in response to CFA alone (control) or a preparation containing both CFA and MOG_{35-55}. These darkfield photomicrographs of nuclear emulsion-dipped sections show positive hybridization signal in cells lining the blood vessels as early as 4 days after immunization with the MOG peptide, although a moderate signal was also found in animals challenged with the CFA preparation alone. Note that CFA-induced IκBα expression increased at day 8 and returned to basal levels at time 3 weeks. This contrasts with the IκBα mRNA levels that spread within the brain parenchyma of animals that received the antigenic peptide MOG and were killed 3 weeks thereafter (bottom right). bv, blood vessels; chp, choroid plexus; hip, hippocampal formation; IC, inflammatory cluster; V3, third ventricle. Magnification, 4× (scale bar, 250 μm).

parenchyma. The signal for the gene encoding the proinflammatory cytokine diffused across the brain and in regions lining the external edge of spinal cord of mice killed 3 weeks after being immunized with MOG_{35-55} (Figure 5, bottom panels).

Transcriptional activation of the MCP-1 gene occurred in the leptomeninges, chp, and few blood vessels as soon as 8 days during the course of EAE (Figure 6, top panels). Two days later, agglomeration of MCP-1-expressing cells were found in the brain parenchyma, although this phenomenon was rare and restricted to few isolated clusters in the cerebral cortex. The signal became very intense across the brain and the spinal cord of mice killed 3 weeks after immunization with MOG_{35-55} (Figure 6, bottom panels).

Discussion

The present data provide solid evidence that receptors of the innate immune system (TLR2 and CD14) are induced in the cerebral tissue in the course of EAE, an animal model of MS. Transcriptional activation is taking place as early as 8 to 10 days after the booster MOG_{35-55} peptide injection in barrier-associated regions and within few isolated microglial cells across the CNS. The signal for both receptor transcripts became very strong within the brain and spinal cord parenchyma 3 weeks after immunization with MOG_{35-55}. A parallel

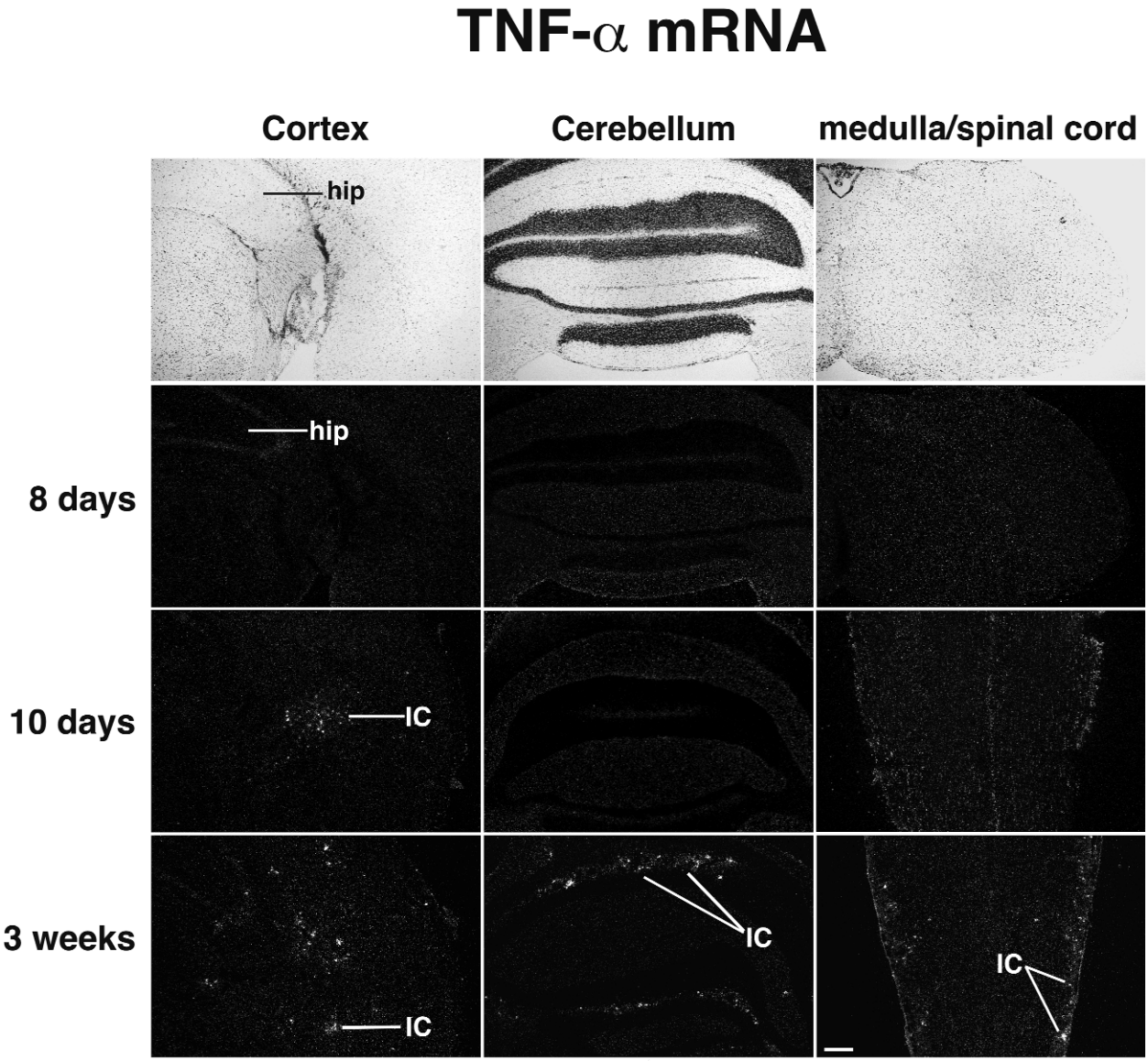

Figure 5. Time-related induction of the mRNA encoding TNF-α in the CNS of EAE mice. These darkfield photomicrographs of nuclear emulsion-dipped sections depict localized hybridization signal in the cerebral cortex (left column), cerebellum (middle column) and the caudal medulla/spinal cord (right column) 8, 10, and 21 days after MOG immunization. Although the in situ hybridization failed to detect convincing signal in the CNS of EAE mice at day 8, few clusters of TNF-α-expressing cells were found in the cortex of animals killed 10 days after the treatment. The signal increased and numerous clusters of positive cells were observed in the brain and spinal cord at time 3 weeks (bottom panels). IC, inflammatory cluster; hip, hippocampal formation. Magnification, 4× (scale bar, 250 μm).

increase in the expression of IκBα, MCP-1, and TNF-α mRNA occurred in TLR2-expressing regions. The early induction of molecules that are currently well-characterized as receptors recognizing bacterial cell wall components suggests that these molecules may play an initiating role in orchestrating the innate immune response in the CNS of EAE animals. The question arises as to whether these receptors are functionally activated in the absence of their known ligands, or if other endogenous ligands may exist.

Although TLR4 plays a key role in the innate immune response to Gram-negative bacteria, TLR2 recognizes various fungal, Gram-positive, and mycobacterial elements (for review, see 3). The broad spectrum of components recognized by TLR2, together with the existence of an additional nine TLRs, suggests that

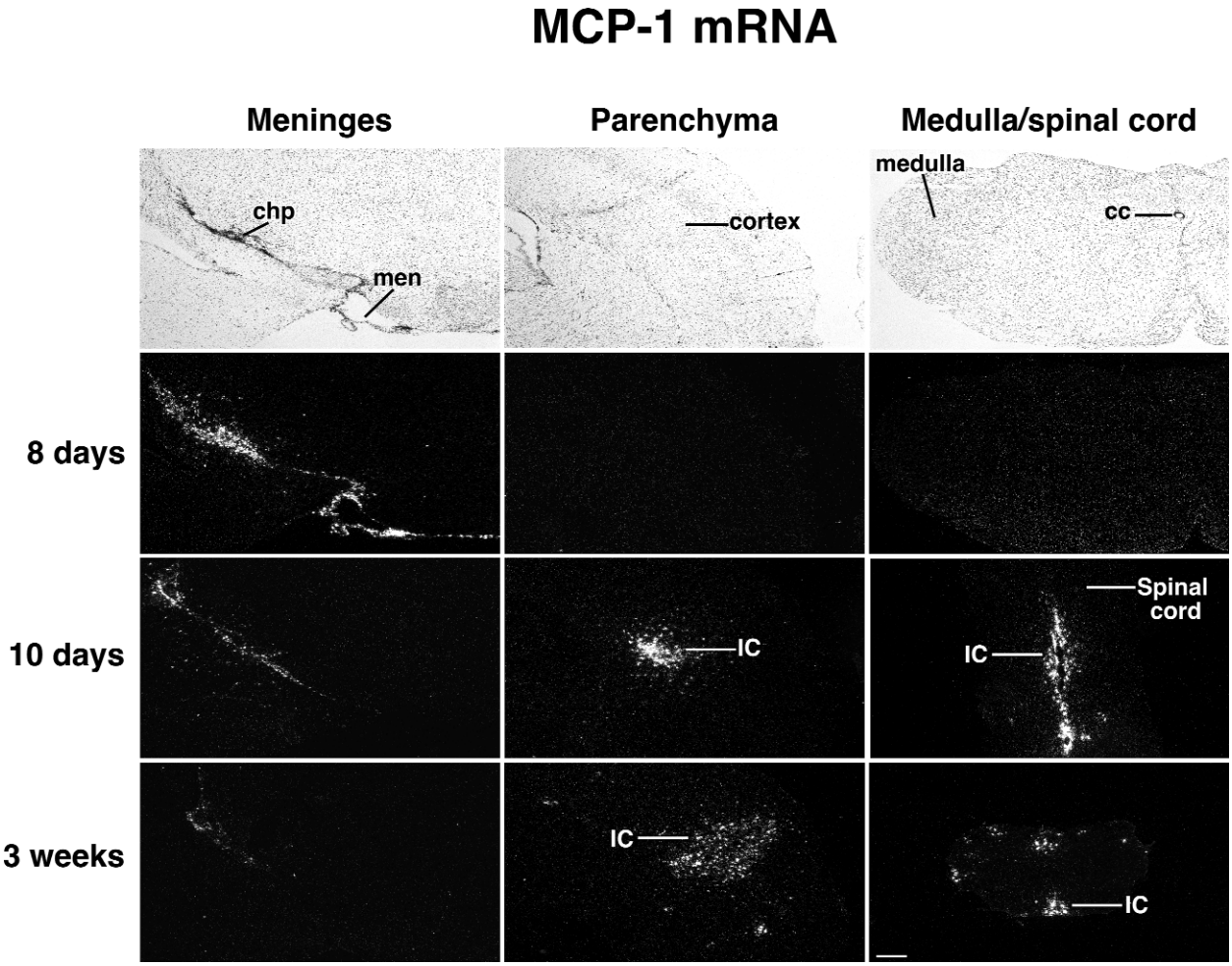

Figure 6. Robust transcriptional activation of the chemokine MCP-1 in the brain and spinal cord of EAE mice. These darkfield photomicrographs of 20-μm sections dipped into NTB2 emulsion depict a positive hybridization signal for MCP-1 mRNA in the choroid plexus and cells lining the leptomeninges underneath the cerebral peduncle at day 8 in the course of EAE. Isolated parenchymal clusters of MCP-1-expressing cells occurred in the cerebral cortex and the spinal cord of mice killed 10 days after MOG immunization. At 3 weeks, the signal became very intense and numerous clusters of positive cells were found across the brain and spinal cord (bottom panels). Magnification, 10× (scale bar, 250 μm).

these receptors participate in a complex pattern of recognition (15). Surprisingly however, transcription of the gene encoding TLR2 is triggered in the brain of EAE mice, the animal model of MS. Of interest is the presence of gram-positive receptors within microglia. These cells are believed to act as the antigen-presenting cells (APCs) that can process and present endogenous self epitopes to autoantigen-specific T cells, and thus provide a mechanistic basis by which epitope spreading occurs (7). In the same line, the lack of expression of CD40 by parenchymal microglia diminishes the intensity and duration of MOG-induced EAE and also reduces the degree of inflammatory cell infiltrates into the CNS (2). Moreover, encephalitogenic T cells that enter a CNS environment in which CD40 is absent from parenchymal microglia do not have the ability to elicit chemokine expression within the CNS (2). It is tempting to propose that activation of the innate immune system within the resident population of immune cells in the CNS plays a key role in this process in which TLR2 and CD14 may be integrative partners. Obviously, transient induction of the cerebral innate immunity during acute endotoxemia is unlikely to be associated with neurodegeneration, but chronic and sustained expression of key components of this system may have detrimental consequences for the neuronal elements.

A strong increase in CD14 transcription was detected in the CNS of mice immunized with MOG, but the

technique used in the present study failed to detect induction of the gene encoding TLR4 (data not shown). In this regard, CD14-expressing cells were clearly devoid of TLR4 transcript in microglia across the brain parenchyma during moderate and severe endotoxemia (10). It is possible that TLR4 is the recognizing molecule for Gram-negative bacterial components only in response to systemic infection, whereas TLR2 and CD14 have a more complex role in the proinflammatory signal transduction events in the brain parenchyma. Both TLR2 and CD14 receptors cooperate in macrophages to recognize and engage the proinflammatory signal transduction pathways and cytokine expression in presence of leptospiral outer membrane constituents (18). This collaboration between these innate receptors may be an essential characteristic of the cerebral innate immune system that can be activated in an acute or chronic manner depending on the insults and antigens. Although we are still at the embryonic stage of understanding cerebral innate immune system, the chronic expression of TLR2 and CD14 within the parenchymal microglial cells may be the key link between the innate and adaptive immunity that is essential for the clinical course of EAE.

In conclusion, there is a robust and sustained expression of the gene encoding TLR2, CD14 and proinflammatory molecules in the CNS of mice immunized with the antigenic peptide MOG. Therefore, these receptors are not only involved in pathogen recognition and signaling, but also in the course of EAE, cerebral inflammation, and possibly, in the etiology of MS. These molecules are likely to be part of the endogenous "alarm" signals originating from alerted microglial cells within specific regions of the spinal cord and brain. Better understanding these events will not only underline the fundamental role(s) of this newly discovered system, but may help finding new avenues to better control the cerebral innate immunity that may have a leading role in brain disorders that have an immune etiology or those exacerbated by an inappropriate control of the cerebral inflammatory response.

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